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☐ 1. Document ID: US 3582191 A

L12: Entry 1 of 1

File: USPT

Jun 1, 1971

US-PAT-NO: 3582191

DOCUMENT-IDENTIFIER: US 3582191 A

TITLE: THREE-DIMENSIONAL INDIRECT OPHTHALMOSCOPE

DATE-ISSUED: June 1, 1971

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Cohen; David N.	Both of Watertown	MA	02172	
Lambert; Brent W.	Both of Watertown	MA	02172	

US-CL-CURRENT: 351/221; 351/205, 359/880, 600/111, 600/248

Full	Title	Citation	Front	Review	Classification	Date	Reference	Excluded	Unpublished	Claims	RWMC	Draw D
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L9: Entry 1 of 105

File: USPT

Oct 26, 2004

US-PAT-NO: 6808111

DOCUMENT-IDENTIFIER: US 6808111 B2

TITLE: Terminal software architecture for use with smart cards

DATE-ISSUED: October 26, 2004

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kashef; Forough	Fremont	CA		
Billon; Jean-Paul	San Francisco	CA		
Colas; Christophe	Versailles			FR
Nakamura; Lance Shigeo	Aiea	HI		
Sak; Thomas H.	Santa Cruz	CA		

US-CL-CURRENT: 235/380; 235/379, 235/381, 235/383, 705/41, 705/43

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstract	Claims	RMIC	Draw Dg
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☐ 2. Document ID: US 6792111 B1

L9: Entry 2 of 105

File: USPT

Sep 14, 2004

US-PAT-NO: 6792111

DOCUMENT-IDENTIFIER: US 6792111 B1

TITLE: Cryptation system for packet switching networks based on digital chaotic models

DATE-ISSUED: September 14, 2004

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Italia; Francesco	Catania			IT
Fortuna; Luigi	Syracuse			IT
Beritelli; Francesco	Catania			IT
Di Cola; Eusebio	Messina			IT

US-CL-CURRENT: 380/263; 380/206, 380/209, 380/257, 380/45, 713/160, 713/201

Full	Title	Citation	Front	Review	Classification	Date	Reference	Attachments	Claims	KWC	Draw De
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☐ 3. Document ID: US 6766310 B1

L9: Entry 3 of 105

File: USPT

Jul 20, 2004

US-PAT-NO: 6766310

DOCUMENT-IDENTIFIER: US 6766310 B1

TITLE: Neuro-fuzzy network architecture with on-line learning capabilities and corresponding control method

DATE-ISSUED: July 20, 2004

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Occhipinti; Luigi	Ragusa			IT
Di Cola; Eusebio	Messina			IT
Lavorgna; Mario	Bacoli			IT

US-CL-CURRENT: 706/16; 711/149

Full	Title	Citation	Front	Review	Classification	Date	Reference	Attachments	Claims	KWC	Draw De
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☐ 4. Document ID: US 6678670 B2

L9: Entry 4 of 105

File: USPT

Jan 13, 2004

US-PAT-NO: 6678670

DOCUMENT-IDENTIFIER: US 6678670 B2

**\*\* See image for Certificate of Correction \*\***

TITLE: Non-integer order dynamic systems

DATE-ISSUED: January 13, 2004

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Abbisso; Salvatore	Augusta			IT
Caponetto; Riccardo	Catania			IT
Diamante; Olga	Syracuse			IT
Porto; Domenico	Pedara			IT
Cola; Eusebio Di	Messina			IT
Fortuna; Luigi	Syracuse			IT

US-CL-CURRENT: 706/44; 706/41

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Attachments	Claims	KMNC	Draw De
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☐ 5. Document ID: US 6647493 B1

L9: Entry 5 of 105

File: USPT

Nov 11, 2003

US-PAT-NO: 6647493

DOCUMENT-IDENTIFIER: US 6647493 B1

**\*\* See image for Certificate of Correction \*\***

TITLE: Method and system for authentication and electronic signature

DATE-ISSUED: November 11, 2003

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Occhipinti; Luigi	Ragusa			IT
Di Bernardo; Giovanni	Mascalucia			IT
Di Cola; Eusebio	Messina			IT
Caponetto; Riccardo	Catania			IT

US-CL-CURRENT: 713/170; 380/263, 713/168, 713/169

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Attachments	Claims	KMNC	Draw De
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☐ 6. Document ID: US 6634355 B2

L9: Entry 6 of 105

File: USPT

Oct 21, 2003

US-PAT-NO: 6634355

DOCUMENT-IDENTIFIER: US 6634355 B2

TITLE: Single breath induction anesthesia apparatus

DATE-ISSUED: October 21, 2003

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Colas; Marie-Jose	Sherbrooke		J1J 1M1	CA

US-CL-CURRENT: 128/203.12; 128/203.28, 128/205.11, 128/205.24

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Attachments	Claims	KMNC	Draw De
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☐ 7. Document ID: US 6613258 B1

L9: Entry 7 of 105

File: USPT

Sep 2, 2003

US-PAT-NO: 6613258

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DOCUMENT-IDENTIFIER: US 6613258 B1

TITLE: Method for making parts in composite material with thermoplastic matrix

DATE-ISSUED: September 2, 2003

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maison; Serge	Puteaux			FR
Meunier; Serge	Paris			FR
Thibout; Cedric	Toulouse			FR
Mouton; Luc	Vitrolles			FR
Payen; Herve	Velaun			FR
Vautey; Philippe	Rueil Malmaison			FR
Coiffier-Colas; Carole	Rueil Malmaison			FR
Delbez; Joel	Merighac			FR

US-CL-CURRENT: 264/102; 156/189, 156/297, 264/160, 264/248, 264/250, 264/255,  
264/257, 264/258, 264/310, 264/324, 264/339, 264/510, 264/511, 264/512

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstract	Attachment	Claims	KWIC	Draw Data
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☐ 8. Document ID: US 6519655 B1

L9: Entry 8 of 105

File: USPT

Feb 11, 2003

US-PAT-NO: 6519655

DOCUMENT-IDENTIFIER: US 6519655 B1

TITLE: Message preprocessing operations indicated by an associated descriptor read and descriptors belonging to a category of preprocessing descriptors and a category of instruction descriptors

DATE-ISSUED: February 11, 2003

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Pitot; Christian	Boulogne Billancourt			FR
Colas; Gerard	Versailles			FR

US-CL-CURRENT: 710/5; 370/465, 370/471, 370/475, 710/3, 710/4

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstract	Attachment	Claims	KWIC	Draw Data
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☐ 9. Document ID: US 6415190 B1

L9: Entry 9 of 105

File: USPT

Jul 2, 2002

US-PAT-NO: 6415190

DOCUMENT-IDENTIFIER: US 6415190 B1

h e b b g e e e f e c e f b e

TITLE: Method and device for executing by a single processor several functions of different criticality levels, operating with high security

DATE-ISSUED: July 2, 2002

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Colas; Gerard	Versailles			FR
Le Borgne; Olivier	Montigny le Bretonneux			FR
Villard; Robert	Plaisir			FR

US-CL-CURRENT: 700/79; 700/306, 711/163, 714/55, 718/107

Full	Title	Citation	Front	Review	Classification	Date	Reference	Attachments	Attachments	Claims	KWIC	Draw. De
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☐ 10. Document ID: US 6397243 B1

L9: Entry 10 of 105

File: USPT

May 28, 2002

US-PAT-NO: 6397243

DOCUMENT-IDENTIFIER: US 6397243 B1

TITLE: Method and device for processing several technical applications each provided with its particular security

DATE-ISSUED: May 28, 2002

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Colas; Gerard	Versailles			FR
Guedou; Philippe	Rambouillet			FR
Le Borgne; Olivier	Montigny le Bretonneux			FR
Rowenczyn; Jean-Jacques	Ris-Orangis			FR

US-CL-CURRENT: 718/100; 710/47, 710/48, 710/52, 712/35, 712/36

Full	Title	Citation	Front	Review	Classification	Date	Reference	Attachments	Attachments	Claims	KWIC	Draw. De
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☐ 1. Document ID: US 6399296 B1

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File: USPT

Jun 4, 2002

US-PAT-NO: 6399296

DOCUMENT-IDENTIFIER: US 6399296 B1

**\*\* See image for Certificate of Correction \*\***

TITLE: Interaction trap systems for detecting protein interactions

DATE-ISSUED: June 4, 2002

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
<u>Brent</u> ; Roger	Cambridge	MA		
McCoy; John M.	Reading	MA		
Jessen; Timm H.	Bad Soden			DE
Xu; Chanxing Wilson	Boston	MA		

US-CL-CURRENT: 435/6; 435/4, 435/7.1, 435/7.8, 436/501

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Attachment	Claims	KWIC	Draw. Des
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☐ 2. Document ID: US 6242183 B1

L14: Entry 2 of 3

File: USPT

Jun 5, 2001

US-PAT-NO: 6242183

DOCUMENT-IDENTIFIER: US 6242183 B1

TITLE: Interaction trap systems for detecting protein interactions

DATE-ISSUED: June 5, 2001

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
<u>Brent</u> ; Roger	Cambridge	MA		
McCoy; John M.	Reading	MA		
Jessen; Timm H.	Bad Soden			DE

US-CL-CURRENT: 435/6; 435/7.1

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Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstracts	Abstracts	Claims	KWIC	Draw. De
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☐ 3. Document ID: US 6004746 A

L14: Entry 3 of 3

File: USPT

Dec 21, 1999

US-PAT-NO: 6004746

DOCUMENT-IDENTIFIER: US 6004746 A

TITLE: Interaction trap systems for detecting protein interactions

DATE-ISSUED: December 21, 1999

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Brent; Roger	Cambridge	MA		
McCoy; John M.	Reading	MA		
Jessen; Timm H.	Bad Soden			DE

US-CL-CURRENT: 435/6; 435/254.21, 435/325, 435/4

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstracts	Abstracts	Claims	KWIC	Draw. De
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File: USPT

Jun 4, 2002

DOCUMENT-IDENTIFIER: US 6399296 B1

**\*\* See image for Certificate of Correction \*\***

TITLE: Interaction trap systems for detecting protein interactions

Abstract Text (1):

Disclosed herein is a method of determining whether a first protein is capable of physically interacting with a second protein, involving: (a) providing a host cell which contains (i) a reporter gene operably linked to a protein binding site; (ii) a first fusion gene which expresses a first fusion protein, the first fusion protein including the first protein covalently bonded to a binding moiety which is capable of specifically binding to the protein binding site; and (iii) a second fusion gene which expresses a second fusion protein, the second fusion protein including the second protein covalently bonded to a gene activating moiety and being conformationally-constrained; and (b) measuring expression of the reporter gene as a measure of an interaction between the first and the second proteins. Also disclosed are methods for assaying protein interactions, and identifying antagonists and agonists of protein interactions.

INVENTOR (1):

Brent; Roger

Brief Summary Text (5):

Accordingly, in one aspect, the invention features a method of determining whether a first protein is capable of physically interacting with a second protein. The method includes (a) providing a host cell which contains (i) a reporter gene operably linked to a DNA-binding-protein recognition site; (ii) a first fusion gene which expresses a first fusion protein, the first fusion protein comprising the first protein covalently bonded to a binding moiety which is capable of specifically binding to the DNA-binding-protein recognition site; and (iii) a second fusion gene which expresses a second fusion protein, the second fusion protein including the second protein covalently bonded to a gene activating moiety and being conformationally-constrained; and (b) measuring expression of the reporter gene as a measure of an interaction between the first and said second proteins.

Brief Summary Text (6):

Preferably, the second protein is a short peptide of at least 6 amino acids in length and is less than or equal to 60 amino acids in length; includes a randomly generated or intentionally designed peptide sequence; includes one or more loops; or is conformationally-constrained as a result of covalent bonding to a conformation-constraining protein, e.g., thioredoxin or a thioredoxin-like molecule. Where the second protein is covalently bonded to a conformationally constraining protein the invention features a polypeptide wherein the second protein is embedded within the conformation-constraining protein to which it is covalently bonded. Where the conformation-constraining protein is thioredoxin, the invention also features an additional method which includes a second protein which is conformationally-constrained by disulfide bonds between cysteine residues in the amino-terminus and in the carboxy-terminus of the second protein.

Brief Summary Text (7):

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In another aspect, the invention features a method of detecting an interacting protein in a population of proteins, comprising: (a) providing a host cell which contains (i) a reporter gene operably linked to a DNA-binding-protein recognition site; and (ii) a fusion gene which expresses a fusion protein, the fusion protein including a test protein covalently bonded to a binding moiety which is capable of specifically binding to the DNA-binding-protein recognition site; (b) introducing into the host cell a second fusion gene which expresses a second fusion protein, the second fusion protein including one of said population of proteins covalently bonded to a gene activating moiety and being conformationally-constrained; and (c) measuring expression of the reporter gene. Preferably, the population of proteins includes short peptides of between 1 and 60 amino acids in length.

Brief Summary Text (8):

The invention also features a method of detecting an interacting protein within a population wherein the population of proteins is a set of randomly generated or intentionally designed peptide sequences, or where the population of proteins is conformationally-constrained by covalently bonding to a conformation-constraining protein. Preferably, where the population of proteins is conformationally-constrained by covalent bonding to a conformation-constraining protein, the population of proteins is embedded within the conformation-constraining protein. The invention further features a method of detecting an interacting protein within a population wherein the conformation-constraining protein is thioredoxin. Preferably, the population of proteins is inserted into the active site loop of the thioredoxin.

Brief Summary Text (9):

The invention further features a method wherein each of the population of proteins is conformationally-constrained by disulfide bonds between cysteine residues in the amino-terminus and in the carboxy-terminus of said protein.

Brief Summary Text (12):

In another related aspect, the invention features a method of identifying a candidate interactor. The method includes (a) providing a reporter gene operably linked to a DNA-binding-protein recognition site; (b) providing a first fusion protein, which includes a first protein covalently bonded to a binding moiety which is capable of specifically binding to the DNA-binding-protein recognition site; (c) providing a second fusion protein, which includes a second protein covalently bonded to a gene activating moiety and being conformationally-constrained, the second protein being capable of interacting with said first protein; (d) contacting said candidate interactor with said first protein and/or said second protein; and (e) measuring expression of said reporter gene.

Brief Summary Text (15):

In a preferred embodiment, the candidate interactor is conformationally-constrained and may include one or more loops. Where the candidate interactor is an antagonist, reporter gene expression is reduced. Where the candidate interactor is an agonist, reporter gene expression is increased. The candidate interactor is a member selected from the group consisting of proteins, polynucleotides, and small molecules. In addition, a candidate interactor can be encoded by a member of a cDNA or synthetic DNA library. Moreover, the candidate interactor can be a mutated form of said first fusion protein or said second fusion protein.

Brief Summary Text (16):

In a preferred embodiment of any of the above aspects, the candidate interactor is isolated in vitro and shown to function in vivo, i.e., as a conformationally constrained intracellular peptide.

Brief Summary Text (17):

In a related aspect, the invention features a population of eukaryotic cells, each cell having a recombinant DNA molecule encoding a conformationally-constrained

intracellular peptide, there being at least 100 different recombinant molecules in the population, each molecule being in at least one cell of said population.

Brief Summary Text (18):

Preferably, the intracellular peptides within the population of cells are conformationally-constrained because they are covalently bonded to a conformation-constraining protein.

Brief Summary Text (19):

In preferred embodiments the intracellular peptide is embedded within the conformation-constraining protein, preferably thioredoxin; the intracellular peptide is conformationally-constrained by disulfide bonds between cysteine residues in the amino-terminus and in the carboxy-terminus of said second protein; the intracellular peptide includes one or more loops; the population of eukaryotic cells are yeast cells; the recombinant DNA molecule further encodes a gene activating moiety covalently bonded to said intracellular peptide; and/or the intracellular peptide physically interacts with a second recombinant protein inside said eukaryotic cells.

Brief Summary Text (20):

In another aspect, the invention features a method of assaying an interaction between a first protein and a second protein. The method includes: (a) providing a reporter gene operably linked to a DNA-binding-protein recognition site; (b) providing a first fusion protein including a first protein covalently bonded to a binding moiety which is capable of specifically binding to the DNA-binding-protein recognition site; (c) providing a second fusion protein including a second protein which is conformationally constrained (and may include one or more loops) and is covalently bonded to a gene activating moiety; (d) combining the reporter gene, the first fusion protein, and the second fusion protein; and (e) measuring expression of the reporter gene.

Brief Summary Text (21):

In a preferred embodiment, the invention further features a method of assaying the interaction between two proteins wherein the first fusion protein is provided by providing a first fusion gene which expresses the first fusion protein and wherein the second fusion protein is provided by providing a second fusion gene which expresses the second fusion protein. In another preferred embodiment, the interaction is assayed in vitro and shown to function in vivo, i.e., as a conformationally constrained intracellular peptide.

Brief Summary Text (22):

In yet other aspects, the invention features a protein including the sequence Leu-Val-Cys-Lys-Ser-Tyr-Arg-Leu-Asp-Trp-Glu-Ala-Gly-Ala-Leu-Phe-Arg-Ser-Leu-Phe (SEQ ID NO: 1), preferably conformationally-constrained; a protein including the sequence Met-Val-Val-Ala-Ala-Glu-Ala-Val-Arg-Thr-Val-Leu-Leu-Ala-Asp-Gly-Gly-Asp-Val-Thr (SEQ ID NO: 2); preferably conformationally-constrained; a protein including the sequence Pro-Asn-Trp-Pro-His-Gln-Leu-Arg-Val-Gly-Arg-Val-Leu-Trp-Glu-Arg-Leu-Ser-Phe-Glu (SEQ ID NO: 3), preferably conformationally-constrained; a protein including the sequence Ser-Val-Arg-Met-Arg-Tyr-Gly-Ile-Asp-Ala-Phe-Phe-Asp-Leu-Gly-Gly-Leu-Leu-His-Gly (SEQ ID NO: 9), preferably conformationally-constrained; a protein including the sequence Glu-Leu-Arg-His-Arg-Leu-Gly-Arg-Ala-Leu-Ser-Glu-Asp-Met-Val-Arg-Gly-Leu-Ala-Trp-Gly-Pro-Thr-Ser-His-Cys-Ala-Thr-Val-Pro-Gly-Thr-Ser-Asp-Leu-Trp-Arg-Val-Ile-Arg-Phe-Leu (SEQ ID NO: 10), preferably conformationally-constrained; a protein including the sequence Tyr-Ser-Phe-Val-His-His-Gly-Phe-Phe-Asn-Phe-Arg-Val-Ser-Trp-Arg-Glu-Met-Leu-Ala (SEQ ID NO: 11), preferably conformationally-constrained; a protein including the sequence Gln-Val-Trp-Ser-Leu-Trp-Ala-Leu-Gly-Trp-Arg-Trp-Leu-Arg-Arg-Tyr-Gly-Trp-Asn-Met (SEQ ID NO: 12), preferably conformationally-constrained; a protein including the sequence Trp-Arg-Arg-Met-Glu-Leu-Asp-Ala-Glu-Ile-Arg-Trp-Val-Lys-Pro-Ile-Ser-Pro-Leu-Glu (SEQ ID NO: 13), preferably conformationally-constrained; a protein including the sequence

Trp-Ala-Glu-Trp-Cys-Gly-Pro-Val-Cys-Ala-His-Gly-Ser-Arg-Ser-Leu-Thr-Leu-Leu-Thr-Lys-Tyr-His-Val-Ser-Phe-Leu-Gly-Pro-Cys-Lys-Met-Ile-Ala-Pro-Ile-Leu-Asp (SEQ ID NO:17), preferably conformationally-constrained; a protein including the sequence Leu-Val-Cys-Lys-Ser-Tyr-Arg-Leu-Asp-Trp-Glu-Ala-Gly-Ala-Leu-Phe-Arg-Ser-Leu-Phe (SEQ ID NO: 18), preferably conformationally-constrained; a protein including the sequence Tyr-Arg-Trp-Gln-Gln-Gly-Val-Val-Pro-Ser-Asn-Trp-Ala-Ser-Cys-Ser-Phe-Arg-Cys-Gly (SEQ ID NO: 19), preferably conformationally-constrained; a protein including the sequence Ser-Ser-Phe-Ser-Leu-Trp-Leu-Leu-Met-Val-Lys-Ser-Ile-Lys-Arg-Ala-Ala-Trp-Glu-Leu-Gly-Pro-Ser-Ser-Ala-Trp-Asn-Thr-Ser-Gly-Trp-Ala-Ser-Leu-Ala-Asp-Phe-Tyr (SEQ ID NO: 20) preferably conformationally-constrained; a protein including the sequence Arg-Val-Lys-Leu-Gly-Tyr-Ser-Phe-Trp-Ala-Gln-Ser-Leu-Leu-Arg-Cys-Ile-Ser-Val-Gly (SEQ ID NO: 21), preferably conformationally-constrained; a protein including the sequence Gln-Leu-Tyr-Ala-Gly-Cys-Tyr-Leu-Gly-Val-Val-Ile-Ala-Ser-Ser-Leu-Ser-Ile-Arg-Val (SEQ ID NO: 22), preferably conformationally-constrained; a protein including the sequence Gln-Gln-Arg-Phe-Val-Phe-Ser-Pro-Ser-Trp-Phe-Thr-Cys-Ala-Gly-Thr-Ser-Asp-Phe-Trp-Gly-Pro-Glu-Pro-Leu-Phe-Asp-Trp-Thr-Arg-Asp (SEQ ID NO: 23), preferably conformationally-constrained; a protein including the sequence Arg-Pro-Leu-Thr-Gly-Arg-Trp-Val-Val-Trp-Gly-Arg-Arg-His-Glu-Glu-Cys-Gly-Leu-Thr (SEQ ID NO: 24), preferably conformationally-constrained; a protein including the sequence Pro-Val-Cys-Cys-Met-Met-Tyr-Gly-His-Arg-Thr-Ala-Pro-His-Ser-Val-Phe-Asn-Val-Asp (SEQ ID NO: 25), preferably conformationally-constrained; a protein including the sequence Trp-Ser-Pro-Glu-Leu-Leu-Arg-Ala-Met-Val-Ala-Phe-Arg-Trp-Leu-Leu-Glu-Arg-Arg-Pro (SEQ ID NO: 26); and substantially pure DNA encoding the immediately foregoing proteins.

#### Brief Summary Text (23):

The invention also includes novel proteins and other candidate interactors identified by the foregoing methods. It will be appreciated that these proteins and candidate interactors may either increase or decrease reporter gene activity and that these changes in activity may be measured using assays described herein or known in the art. Also included in the invention are methods for using conformationally constrained interactor proteins. For example, the conformationally constrained proteins of the invention may be used as reagents in assays for protein detection that involve formation of a complex between the conformationally constrained protein and a protein of interest to which it specifically binds, followed by complex detection (for example, by an immunoprecipitation, Western blot, or affinity column technique that utilizes the conformationally constrained protein as the complex-forming reagent).

#### Brief Summary Text (24):

Finally, the invention features a method of assaying an interaction between a first protein and a second protein, involving: (a) providing the first protein; (b) providing a fusion protein including the second protein, the second protein being conformationally-constrained; (c) contacting the first protein with the fusion protein under conditions which allow complex formation; (d) detecting the complex as an indication of an interaction; and (e) determining whether the first protein interacts with the fusion protein inside a cell.

#### Brief Summary Text (26):

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins or proteins which include transcriptional activation domains) are bound to the regulatory sequence(s).

#### Brief Summary Text (27):

By "covalently bonded" is meant that two domains are joined by covalent bonds, directly or indirectly. That is, the "covalently bonded" proteins or protein moieties may be immediately contiguous or may be separated by stretches of one or more amino acids within the same fusion protein.

Brief Summary Text (30):

By a "binding moiety" is meant a stretch of amino acids which is capable of directing specific polypeptide binding to a particular DNA sequence (i.e., a "DNA-binding-protein recognition site").

Brief Summary Text (33):

By "conformationally-constrained" is meant a protein that has reduced structural flexibility because its amino and carboxy termini are fixed in space. As a result of this constraint, the protein may form "loops" (i.e., regions of amino acids of any shape which extend away from the constrained amino and carboxy termini). Preferably, the conformationally-constrained protein is displayed in a structurally rigid manner. Conformational constraint according to the invention may be brought about by exploiting the disulfide-bonding ability of a natural or recombinantly-introduced pair of cysteine residues, one residing at or near the amino-terminal end of the protein of interest and the other at or near the carboxy-terminal end. Alternatively, conformational constraint may be facilitated by embedding the protein of interest within a conformation-constraining protein.

Brief Summary Text (34):

By "conformation-constraining protein" is meant any peptide or polypeptide which is capable of reducing the flexibility of another protein's amino and/or carboxy termini. Preferably, such proteins provide a rigid scaffold or platform for the protein of interest. In addition, such proteins preferably are capable of providing protection from proteolytic degradation and the like, and/or are capable of enhancing solubility. Examples of conformation-constraining proteins include thioredoxin and other thioredoxin-like proteins, nucleases (e.g., RNase A), proteases (e.g., trypsin), protease inhibitors (e.g., bovine pancreatic trypsin inhibitor), antibodies or structurally-rigid fragments thereof, conotoxins, and the pleckstrin homology domain. A conformation-constraining peptide can be of any appropriate length and can even be a single amino acid residue.

Brief Summary Text (35):

"Thioredoxin-like proteins" are defined herein as amino acid sequences substantially similar, e.g., having at least 18% homology, with the amino acid sequence of E. coli thioredoxin over an amino acid sequence length of 80 amino acids. Alternatively, a thioredoxin-like DNA sequence is defined herein as a DNA sequence encoding a protein or fragment of a protein characterized by having a three dimensional structure substantially similar to that of human or E. coli thioredoxin, e.g., glutaredoxin and optionally by containing an active-site loop. The DNA sequence of glutaredoxin is an example of a thioredoxin-like DNA sequence which encodes a protein that exhibits such substantial similarity in three-dimensional conformation and contains a Cys . . . Cys active-site loop. The amino acid sequence of E. coli thioredoxin is described in Eklund et al., EMBO J. 3:1443-1449 (1984). The three-dimensional structure of E. coli thioredoxin is depicted in FIG. 2 of Holmgren, J. Biol. Chem. 264:13963-13966 (1989). A DNA sequence encoding the E. coli thioredoxin protein is set forth in Lim et al., J. Bacteriol., 163:311-316 (1985). The three dimensional structure of human thioredoxin is described in Forman-Kay et al., Biochemistry 30:2685-98 (1991). A comparison of the three dimensional structures of E. coli thioredoxin and glutaredoxin is published in Xia, Protein Science 1:310-321 (1992). These four publications are incorporated herein by reference for the purpose of providing information on thioredoxin-like proteins that is known to one of skill in the art. Examples of thioredoxin-like proteins are described herein.

Brief Summary Text (37):

"Compounds" include small molecules, generally under 1000 MW, carbohydrates, polynucleotides, lipids, and the like.

Brief Summary Text (41):

By "intracellular" is meant that the peptide is localized inside the cell, rather

than on the cell surface.

Brief Summary Text (45):

In addition, the claimed methods make use of conformationally-constrained proteins (i.e., proteins with reduced flexibility due to constraints at their amino and carboxy termini). Conformational constraint may be brought about by embedding the protein of interest within a conformation-constraining protein (i.e., a protein of appropriate length and amino acid composition to be capable of locking the candidate interacting protein into a particular three-dimensional structure). Examples of conformation-constraining proteins include, but are not limited to, thioredoxin (or other thioredoxin-like proteins), nucleases (e.g., RNase A), proteases (e.g., trypsin), protease inhibitors (e.g., bovine pancreatic trypsin inhibitor), antibodies or structurally-rigid fragments thereof, conotoxins, and the pleckstrin homology domain.

Brief Summary Text (46):

Alternatively, conformational constraint may be accomplished by exploiting the disulfide-bonding ability of a natural or recombinantly-introduced pair of cysteine residues, one residing at the amino terminus of the protein of interest and the other at its carboxy terminus. Such disulfide bonding locks the protein into a rigid and therefore conformationally-constrained loop structure. Disulfide bonds between amino-terminal and carboxy-terminal cysteines may be formed, for example, in the cytoplasm of E. coli trxB mutant strains. Under some conditions disulfide bonds may also form within the cytoplasm and nucleus of higher organisms harboring equivalent mutations, for example, an S. cerevisiae YTR4.sup.- mutant strain (Furter et al., Nucl Acids Res. 14:6357-6373, 1986; GenBank Accession Number P29509). In addition, the thioredoxin fusions described herein (trxA fusions) are amenable to this alternative means of introducing conformational constraint, since the cysteines at the base of peptides inserted within the thioredoxin active-site loop are at a proper distance from one another to form disulfide bonds under appropriate conditions.

Brief Summary Text (47):

Conformationally-constrained proteins as candidate interactors are useful in the invention because they are amenable to tertiary structural analysis, thus facilitating the design of simple organic molecule mimetics with improved pharmacological properties. For example, because thioredoxin has a known structure, the protein structure between the conformationally constrained regions may be more easily solved using methods such as NMR and X-ray difference analysis. Certain conformation-constraining proteins also protect the embedded protein from cellular degradation and/or increase the protein's solubility, and/or otherwise alter the capacity of the candidate interactor to interact.

Brief Summary Text (48):

Once isolated, interacting proteins can also be analyzed using the interaction trap system, with the signal generated by the interaction being an indication of any change in the proteins' interaction capabilities. In one particular example, an alteration is made (e.g., by standard in vivo or in vitro directed or random mutagenesis procedures) to one or both of the interacting proteins, and the effect of the alteration(s) is monitored by measuring reporter gene expression. Using this technique, interacting proteins with increased or decreased interaction potential are isolated. Such proteins are useful as therapeutic molecules (for example, agonists or antagonists) or, as described above, as models for the design of simple organic molecule mimetics.

Brief Summary Text (49):

Protein agonists and antagonists may also be readily identified and isolated using a variation of the interaction trap system. In particular, once a protein-protein interaction has been recorded, an additional DNA coding for a candidate agonist or antagonist, or preferably, one of a library of potential agonist- or antagonist-



encoding sequences is introduced into the host cell, and reporter gene expression is measured. Alternatively, candidate interactor agonist or antagonist compounds (i.e., including polypeptides as well as non-proteinaceous compounds, e.g., single stranded polynucleotides) are introduced into an in vivo or in vitro interaction trap system according to the invention and their ability to effect reporter gene expression is measured. A decrease in reporter gene expression (compared to a control lacking the candidate sequence or compound) indicates an antagonist. Conversely, an increase in reporter gene expression (compared again to a control) indicates an agonist. Interaction agonists and antagonists are useful as therapeutic agents or as models to design simple mimetics; if desired, an agonist or antagonist protein may be conformationally-constrained to provide the advantages described herein. Particular examples of interacting proteins for which antagonists or agonists may be identified include, but are not limited to, the IL-6 receptor-ligand pair, TGF- $\beta$  receptor-ligand pair, IL-1 receptor-ligand pair and other receptor-ligand interactions, protein kinase-substrate pairs, interacting pairs of transcription factors, interacting components of signal transduction pathways (for example, cytoplasmic domains of certain receptors and G-proteins), pairs of interacting proteins involved in cell cycle regulation (for example, p16 and CDK4), and neurotransmitter pairs.

#### Brief Summary Text (50):

Also included in the present invention are libraries encoding conformationally-constrained proteins. Such libraries (which may include natural as well as synthetic DNA sequence collections) are expressed intracellularly or, optionally, in cell-free systems, and may be used together with any standard genetic selection or screen or with any of a number of interaction trap formats for the identification of interacting proteins, agonist or antagonist proteins, or proteins that endow a cell with any identifiable characteristic, for example, proteins that perturb cell cycle progression. Accordingly, peptide-encoding libraries (either random or designed) can be used in selections or screens which either are or are not transcriptionally-based. These libraries (which preferably include at least 100 different peptide-encoding species and more preferably include 1000, or 100,000 or greater individual species) may be transformed into any useful prokaryotic or eukaryotic host, with yeast representing the preferred host. Alternatively, such peptide-encoding libraries may be expressed in cell-free systems.

#### Detailed Description Text (2):

Applicants have developed a novel interaction trap system for the identification and analysis of conformationally-constrained proteins that either physically interact with a second protein of interest or that antagonize or agonize such an interaction. In one embodiment, the system involves a eukaryotic host strain (e.g., a yeast strain) which is engineered to produce a protein of therapeutic or diagnostic interest as a fusion protein covalently bonded to a known DNA binding domain; this protein is referred to as a "bait" protein because its purpose in the system is to "catch" useful, but as yet unknown or uncharacterized, interacting polypeptides (termed the "prey"; see below). The eukaryotic host strain also contains one or more "reporter genes," i.e., genes whose transcription is detected in response to a bait-prey interaction. Bait proteins, via their DNA binding domain, bind to their specific DNA recognition site upstream of a reporter gene; reporter transcription is not stimulated, however, because the bait protein lacks an activation domain.

#### Detailed Description Text (3):

To isolate DNA sequences encoding novel interacting proteins, members of a DNA expression library (e.g., a cDNA or synthetic DNA library, either random or intentionally biased) are introduced into the strain containing the reporter gene and bait protein; each member of the library directs the synthesis of a candidate interacting protein fused to an invariant gene activation domain tag. Those library-encoded proteins that physically interact with the promoter-bound bait protein are referred to as "prey" proteins. Such bound prey proteins (via their

activation domain tag) detectably activate the expression of the downstream reporter gene and provide a ready assay for identifying a particular DNA clone encoding an interacting protein of interest. In the instant invention, each candidate prey protein is conformationally-constrained (for example, either by embedding the protein within a conformation-constraining protein or by linking together the protein's amino and carboxy termini). Such a protein is maintained in a fixed, three-dimensional structure, facilitating mimetic drug design.

Detailed Description Text (4):

An example of one interaction trap system according to the invention is shown in FIGS. 1A-C. FIG. 1A shows a leucine auxotroph yeast strain containing two reporter genes, LexAop-LEU2 and LexAop-lacZ, and a constitutively expressed bait protein gene. The bait protein (shown as a pentagon) is fused to a DNA binding domain (shown as a circle). The DNA binding protein recognizes and binds a specific DNA-binding-protein recognition site (shown as a solid rectangle) operably-linked to a reporter gene. In FIGS. 1B and 1C, the cells additionally contain candidate prey proteins (candidate interactors) (shown as an empty rectangle in 1B and an empty hexagon in 1C) fused to an activation domain (shown as a solid square); each prey protein is embedded in a conformation-constraining protein (shown as two solid half circles). FIG. 1B shows that if the candidate prey protein does not interact with the transcriptionally-inert LexA-fusion bait protein, the reporter genes are not transcribed; the cell cannot grow into a colony on leu.sup.- medium, and it is white on Xgal medium because it contains no .beta.-galactosidase activity. FIG. 1C shows that, if the candidate prey protein interacts with the bait, both reporter genes are active; the cell forms a colony on leu.sup.- medium, and cells in that colony have .beta.-galactosidase activity and are blue on Xgal medium. Preferably, in this system, the bait protein (i.e., the protein containing a site-specific DNA binding domain) is transcriptionally inert, and the reporter genes (which are bound by the bait protein) have essentially no basal transcription.

Detailed Description Text (8):

Preferably, the bait protein also includes a LexA dimerization domain; this optional domain facilitates efficient LexA dimer formation. Because LexA binds its DNA binding site as a dimer, inclusion of this domain in the bait protein also optimizes the efficiency of operator occupancy (Golemis and Brent, Mol. Cell Biol. 12:3006-3014, (1992)).

Detailed Description Text (16):

In the selection described herein, another DNA construction is utilized which encodes a series of candidate interacting proteins (i.e., prey proteins); each is conformationally-constrained, either by being embedded in a conformation-constraining protein or because the prey protein's amino and carboxy termini are linked (e.g., by disulfide bonding). An exemplary prey protein includes an invariant N-terminal moiety carrying, amino to carboxy terminal, an ATG for protein expression, an optional nuclear localization sequence, a weak activation domain (e.g., the B112 or B42 activation domains of Ma and Ptashne; Cell 51:113, 1987), and an optional epitope tag for rapid immunological detection of fusion protein synthesis. Library sequences, random or intentionally designed synthetic DNA sequences, or sequences encoding conformationally-constrained proteins, may be inserted downstream of this N-terminal fragment to produce fusion genes encoding prey proteins.

Detailed Description Text (18):

Similarly, any number of activation domains may be used for that portion of the prey molecule; such activation domains are preferably weak activation domains, i.e., weaker than the GAL4 activation region II moiety and preferably no stronger than B112 (as measured, e.g., by a comparison with GAL4 activation region II or B112 in parallel .beta.-galactosidase assays using lacZ reporter genes); such a domain may, however, be weaker than B112. In particular, the extraordinary sensitivity of the LEU2 selection scheme allows even extremely weak activation

domains to be utilized in the invention. Examples of other useful weak activation domains include B17, B42, and the amphipathic helix (AH) domains described in Ma and Ptashne (Cell 51:113, 1987), Ruden et al. (Nature 350:426-430, 1991), and Giniger and Ptashne (Nature 330:670, 1987).

Detailed Description Text (19):

The prey proteins, if desired, may include other optional nuclear localization sequences (e.g., those derived from the GAL4 or MAT.alpha.2 genes) or other optional epitope tags (e.g., portions of the c-myc protein or the flag epitope available from Immunex). These sequences optimize the efficiency of the system, but are not required for its operation. In particular, the nuclear localization sequence optimizes the efficiency with which prey molecules reach the nuclear-localized reporter gene construct(s), thus increasing their effective concentration and allowing one to detect weaker protein interactions. The epitope tag merely facilitates a simple immunoassay for fusion protein expression.

Detailed Description Text (22):

According to one embodiment of the present invention, the DNA sequence encoding the prey protein is embedded in a DNA sequence encoding a conformation-constraining protein (i.e., a protein that decreases the flexibility of the amino and carboxy termini of the prey protein). Methods for directly linking the amino and carboxy termini of a protein (e.g., through disulfide bonding of appropriately positioned cysteine residues) are described above. As an alternative to this approach, conformation-constraining proteins may be utilized. In general, conformation-constraining proteins act as scaffolds or platforms, which limit the number of possible three dimensional configurations the peptide or protein of interest is free to adopt. Preferred examples of conformation-constraining proteins are thioredoxin or other thioredoxin-like sequences, but many other proteins are also useful for this purpose. Preferably, conformation-constraining proteins are small in size (generally, less than or equal to 200 amino acids), rigid in structure, of known three dimensional configuration, and are able to accommodate insertions of proteins of interest without undue disruption of their structures. A key feature of such proteins is the availability, on their solvent exposed surfaces, of locations where peptide insertions can be made (e.g., the thioredoxin active-site loop). It is also preferable that conformation-constraining protein producing genes be highly expressible in various prokaryotic and eukaryotic hosts, or in suitable cell-free systems, and that the proteins be soluble and resistant to protease degradation. Examples of conformation-constraining proteins useful in the invention include nucleases (e.g., RNase A), proteases (e.g., trypsin), protease inhibitors (e.g., bovine pancreatic trypsin inhibitor), antibodies or rigid fragments thereof, conotoxins, and the pleckstrin homology domain. This list, however, is not limiting. It is expected that other conformation-constraining proteins having sequences not identified above, or perhaps not yet identified or published, may be useful based upon their structural stability and rigidity.

Detailed Description Text (23):

As mentioned above, one preferred conformation-constraining protein according to the invention is thioredoxin or other thioredoxin-like proteins. As one example of a thioredoxin-like protein useful in this invention, E. coli thioredoxin has the following characteristics. E. coli thioredoxin is a small protein, only 11.7 kD, and can be produced to high levels. The small size and capacity for high level synthesis of the protein contributes to a high intracellular concentration. E. coli thioredoxin is further characterized by a very stable, tight tertiary structure which can facilitate protein purification.

Detailed Description Text (24):

The three dimensional structure of E. coli thioredoxin is known and contains several surface loops, including a distinctive Cys . . . Cys active-site loop between residues Cys.sub.33 and Cys.sub.36 which protrudes from the body of the protein. This Cys . . . Cys active-site loop is an identifiable, accessible surface

loop region and is not involved in interactions with the rest of the protein which contribute to overall structural stability. It is therefore a good candidate as a site for prey protein insertions. Human thioredoxin, glutaredoxin, and other thioredoxin-like molecules also contain this Cys . . . Cys active-site loop. Both the amino- and carboxyl-termini of E. coli thioredoxin are on the surface of the protein and are also readily accessible for fusion construction. E. coli thioredoxin is also stable to proteases, stable in heat up to 80.degree. C. and stable to low pH.

Detailed Description Text (25):

Other thioredoxin-like proteins encoded by thioredoxin-like DNA sequences useful in this invention share homologous amino acid sequences, and similar physical and structural characteristics. Thus, DNA sequences encoding other thioredoxin-like proteins may be used in place of E. coli thioredoxin according to this invention. For example, the DNA sequence encoding other species' thioredoxin, e.g., human thioredoxin, are suitable. Human thioredoxin has a three-dimensional structure that is virtually superimposable on E. coli's three-dimensional structure, as determined by comparing the NMR structures of the two molecules. Forman-Kay et al., Biochem. 30:2685 (1991). Human thioredoxin also contains an active-site loop structurally and functionally equivalent to the Cys . . . Cys active-site loop found in the E. coli protein. It can be used in place of or in addition to E. coli thioredoxin in the production of protein and small peptides in accordance with the method of this invention. Insertions into the human thioredoxin active-site loop and onto the amino terminus may be as well-tolerated as those in E. coli thioredoxin.

Detailed Description Text (26):

Other thioredoxin-like sequences which may be employed in this invention include all or portions of the proteins glutaredoxin and various species' homologs thereof (Holmgren, supra). Although E. coli glutaredoxin and E. coli thioredoxin share less than 20% amino acid homology, the two proteins do have conformational and functional similarities (Eklund et al., EMBO J. 3:1443-1449 (1984)) and glutaredoxin contains an active-site loop structurally and functionally equivalent to the Cys . . . Cys active-site loop of E. coli thioredoxin. Glutaredoxin is therefore a thioredoxin-like molecule as defined herein.

Detailed Description Text (27):

In addition, the DNA sequence encoding protein disulfide isomerase (PDI), or that portion containing the thioredoxin-like domain, and its various species' homologs thereof (Edman et al., Nature 317:267-270 (1985)) may also be employed as a thioredoxin-like DNA sequence, since a repeated domain of PDI shares >30% homology with E. coli thioredoxin and that repeated domain contains an active-site loop structurally and functionally equivalent to the Cys . . . Cys active-site loop of E. coli thioredoxin. The two latter publications are incorporated herein by reference for the purpose of providing information on glutaredoxin and PDI which is known and available to one of skill in the art.

Detailed Description Text (28):

Similarly the DNA sequence encoding phosphoinositide-specific phospholipase C (PI-PLC), fragments thereof, and various species' homologs thereof (Bennett et al., Nature, 334:268-270 (1988)) may also be employed in the present invention as a thioredoxin-like sequence based on the amino acid sequence homology with E. coli thioredoxin, or alternatively based on similarity in three dimensional conformation and the presence of an active-site loop structurally and functionally equivalent to Cys . . . Cys active-site loop of E. coli thioredoxin. All or a portion of the DNA sequence encoding an endoplasmic reticulum protein, ERp72, or various species homologs thereof are also included as thioredoxin-like DNA sequences for the purposes of this invention (Mazzarella et al., J. Biol. Chem. 265:1094-1101 (1990)) based on amino acid sequence homology, or alternatively based on similarity in three dimensional conformation and the presence of an active-site loop structurally and functionally equivalent to Cys . . . Cys active-site loop of E. coli

thioredoxin. Another thioredoxin-like sequence is a DNA sequence which encodes all or a portion of an adult T-cell leukemia-derived factor (ADF) or other species homologs thereof (Wakasugi et al., Proc. Natl. Acad. Sci. USA, 87:8282-8286 (1990)). ADF is now believed to be human thioredoxin. Similarly, the protein responsible for promoting disulfide bond formation in the periplasm of E. coli, the product of the dsbA gene (Bardwell et al., Cell 67:581-89, 1991) also can be considered a thioredoxin-like sequence. The three latter publications are incorporated herein by reference for the purpose of providing information on PI-PLC, ERp72, ADF, and dsbA which are known and available to one of skill in the art.

#### Detailed Description Text (29):

It is expected from the definition of thioredoxin-like sequences used above that other sequences not specifically identified above, or perhaps not yet identified or published, may be useful as thioredoxin-like sequences based on their amino acid sequence homology to E. coli thioredoxin or based on having three dimensional structures substantially similar to E. coli or human thioredoxin and having an active-site loop functionally and structurally equivalent to the Cys . . . Cys active-site loop of E. coli thioredoxin. One skilled in the art can determine whether a molecule has these latter two characteristics by comparing its three-dimensional structure, as analyzed for example by x-ray crystallography or two-dimensional NMR spectroscopy, with the published three-dimensional structure for E. coli thioredoxin and by analyzing the amino acid sequence of the molecule to determine whether it contains an active-site loop that is structurally and functionally equivalent to the Cys . . . Cys active-site loop of E. coli thioredoxin. By "substantially similar" in three-dimensional structure or conformation is meant as similar to E. coli thioredoxin as is glutaredoxin. In addition a predictive algorithm has been described which enables the identification of thioredoxin-like proteins via computer-assisted analysis of primary sequence (Ellis et al., Biochemistry 31:4882-91 (1992)). Based on the above description, one of skill in the art will be able to select and identify, or, if desired, modify, a thioredoxin-like DNA sequence for use in this invention without resort to undue experimentation. For example, simple point mutations made to portions of native thioredoxin or native thioredoxin-like sequences which do not effect the structure of the resulting molecule are alternative thioredoxin-like sequences, as are allelic variants of native thioredoxin or native thioredoxin-like sequences.

#### Detailed Description Text (30):

DNA sequences which hybridize to the sequence for E. coli thioredoxin or its structural homologs under either stringent or relaxed hybridization conditions also encode thioredoxin-like proteins for use in this invention. An example of one such stringent hybridization condition is hybridization at 4.times.SSC at 65.degree. C., followed by a washing in 0.1X SSC at 65.degree. C. for an hour. Alternatively an exemplary stringent hybridization condition is in 50% formamide, 4.times.SSC at 42.degree. C. Examples of non-stringent hybridization conditions are 4.times.SSC at 50.degree. C. or hybridization with 30-40% formamide at 42.degree. C. The use of all such thioredoxin-like sequences are believed to be encompassed in this invention.

#### Detailed Description Text (31):

It may be preferred for a variety of reasons that prey proteins be fused within the active-site loop of thioredoxin or thioredoxin-like molecules. The face of thioredoxin surrounding the active-site loop has evolved, in keeping with the protein's major function as a nonspecific protein disulfide oxido-reductase, to be able to interact with a wide variety of protein surfaces. The active-site loop region is found between segments of strong secondary structure and this provides a rigid platform to which one may tether prey proteins.

#### Detailed Description Text (32):

A small prey protein inserted into the active-site loop of a thioredoxin-like

protein is present in a region of the protein which is not involved in maintaining tertiary structure. Therefore the structure of such a fusion protein is stable. Indeed, E. coli thioredoxin can be cleaved into two fragments at a position close to the active-site loop, and yet the tertiary interactions stabilizing the protein remain.

Detailed Description Text (33):

The active-site loop of E. coli thioredoxin has the sequence NH.sub.2 . . . Cys.sub.33 -Gly-Pro-Cys.sub.36 . . . COOH. Fusing a selected prey protein with a thioredoxin-like protein in the active loop portion of the protein constrains the prey at both ends, reducing the degrees of conformational freedom of the prey protein, and consequently reducing the number of alternative structures taken by the prey. The inserted prey protein is bound at each end by cysteine residues, which may form a disulfide linkage to each other as they do in native thioredoxin and further limit the conformational freedom of the inserted prey.

Detailed Description Text (34):

In addition, by being positioned within the active-site loop, the prey protein is placed on the surface of the thioredoxin-like protein, an advantage for use in screening for bioactive protein conformations and other assays. In general, the utility of thioredoxin or other thioredoxin-like proteins is described in McCoy et al., U.S. Pat. No. 5,270,181 and LaVallie et al., Bio/Technology 11:187-193 (1993). These two references are hereby incorporated by reference.

Detailed Description Text (35):

There now follows a description of thioredoxin interaction trap systems according to the invention. These examples are designed to illustrate, not limit, the invention.

Detailed Description Text (36):

Thioredoxin Interaction Trap System

Detailed Description Text (37):

Interaction trap systems utilizing conformationally-constrained proteins have been developed for the detection of protein interactions, the identification and isolation of proteins participating in such interactions, the identification and isolation of agonists and antagonists of such interactions, and the identification and isolation of interacting peptide aptamers that may be used in protein detection assays in a manner analogous to antibody-type reagents. Exemplary systems are now described.

Detailed Description Text (38):

1. Thioredoxin Interaction Trap with Cdk2 Bait

Detailed Description Text (39):

Progression of eukaryotic cells through the cell cycle requires the coordinated action of a number of regulatory proteins that interact with and regulate the activity of Cdks (Sherr, Cell 79:551-555 (1994)). These modulatory proteins include cyclins, which positively regulate Cdk activity, Cyclin Dependent kinase inhibitors (Ckis), and a number of protein kinases and phosphatases, some of which, such as CAK and Cdc25, positively regulate kinase activity, some of which, such as Weel, inhibit kinase activity, and some of which, such as Cdi1 (Gyuris et al., Cell 75:791-803 (1993)), have effects that are so far unknown (reviewed in Morgan, Nature 374:131-134 (1995)). Cdk2 is thought to be required for higher eukaryotic cells to progress from Gi into S-phase (Fang & Newport, J. Cell Biol. 66:731-742 (1991); Pagano et al., J. Cell Biol. 121:101-111 (1993); van den Heuvel & Harlow, Science 262: 2050-2054 (1993)). Cdk2 kinase activity is positively regulated by Cyclin E and Cyclin A (Koff et al., Science 257:1689-1694 (1992); Dulic et al., Science 257:1958-1961 (1992); Tsai et al., Nature 353:174-7 (1991)) and negatively regulated by p21, p27 and p57 (Harper et al., Cell 75:805-816 (1993); Polyak et

al., Genes Dev. 8:9-22 (1994); Toyoshima & Hunter, Cell 78:67-74 (1994); Matsuoka et al., Genes Dev. 9:660-662 (1995); Lee et al., Genes Dev. 9:639-649 (1995)); in addition, Cdk2 complexes with Cdi1 at the G1 to S transition (Gyuris et al., supra). Here we describe the use of a yeast two-hybrid system to select molecules which recognize Cdk2 from combinatorial libraries.

#### Detailed Description Text (40):

A prey vector is constructed containing the E. coli thioredoxin gene (trxA). pJG 4-4 (Gyuris et al., supra) is used as the vector backbone and cut with EcoRI and XhoI. A DNA fragment encoding the B112 transcription activation domain is obtained by PCR amplification of plasmid LexA-B112 (Doug Ruden, Ph.D. thesis, Harvard University, 1992) and cut with MunI and NdeI. The E. coli trxA gene is excised from the vector pALTRXA-781 (U.S. Pat. No. 5,292,646; InVitrogen Corp., San Diego, Calif.) by digestion with NdeI and SalI. The trxA and B112 fragments are then ligated by standard techniques into the EcoRI/XhoI-cut pJG 4-4 backbone, forming pYENAEtrX. This vector encodes a fusion protein comprising the SV40 nuclear localization domain, the B112 transcription activation domain, an hemagglutinin epitope tag, and E. coli thioredoxin (FIG. 2).

#### Detailed Description Text (41):

Peptide libraries are constructed as follows. The DNA oligomer 5' GACTGACTGGTCCG (NNK).sub.20 GGTCCTCAGTCAGTCAG 3' (with N=A, C, G, T and K=G, T) (SEQ ID NO: 4) is synthesized and annealed to the second oligomer (5' CTGACTGACTGAGGACC 3') (SEQ ID NO: 5) in order to form double stranded DNA at the 3' end of the first oligomer. The second strand is enzymatically completed using Klenow enzyme, priming synthesis with the second oligomer. The product is cleaved with AvaII, and inserted into RsrII cut pYENAEtrX. After ligation, the construct is used to transform E. coli by standard methods (Ausubel et al., Current Protocols in Molecular Biology, (Greene and Wiley-interscience, New York, 1987-1994)). The library contained 2.9.times.10.sup.9 members, of which more than 10.sup.9 directed the synthesis of peptides. Twenty-mers were chosen as preferred peptides because they were long enough to fold into many different patterns of shape and charge and short enough that many of the encoding oligonucleotides lacked stop codons. Because of the presence of fortuitous restriction sites in some coding oligonucleotides and because some library members contained double inserts, approximately one fifth of the constrained peptides were longer or shorter than unit length.

#### Detailed Description Text (42):

To screen for interacting peptides or "aptamers," 100 .mu.g of the library was used to transform the yeast strain EGY48 (Mata his3 leu2::2Lexop-LEU2 ura3 trp1 LYS2; Gyuris et al., supra). This strain also contained the reporter plasmid pSH 18-34, a pLR1.DELTA.1 derivative, containing the yeast 2 .mu. replication origin, the URA3 gene, and a GAL1-lacZ reporter gene with the GAL1 upstream regulatory elements replaced with 4 colE1 LexA operators (West et al., Mol. Cell Biol. 4:2467, 1984; Ebina et al., J. Biol. Chem. 258:13258, 1983; Hanes and Brent, Cell 57:1275, 1989), as well as the bait vector pLexA202-Cdk2 (Cdk2 encodes the human cyclin dependent kinase 2, an essential cell cycle enzyme) (Gyuris et al., supra; Tsai et al., Oncogene 8:1593, 1993). About 2.5.times.10.sup.6 transformants are obtained and pooled. The first selection step, growth on leucine-deficient medium after induction with 2% galactose/1% raffinose (Gyuris et al., supra; Guthrie and Fink, Guide to Yeast Genetics and Molecular Biology, Vol. 194, 1991), was performed with an 8-fold redundancy (20.times.10.sup.6 cfu) of the library in yeast, and about 900 colonies were obtained after growth at 30.degree. C. for 5 days. The 300 largest colonies were streak purified and tested for the galactose-dependent expression of the LEU2 gene product and of .beta.-galactosidase (encoded by pSH 18-34), the latter giving rise to blue yeast colonies in the presence of Xgal in the medium (Ausubel et al., supra). Thirty-three colonies fulfilled these requirements which, after sequencing, included 14 different clones, all of which bound specifically to a LexA-Cdk2 bait but not to LexA or to a LexA-Cdk3 bait (Finley et al., Proc. Natl. Acad. Sci. USA 91:12980-12984 (1994)). The strength of binding was judged according

to the intensity of the blue color formed by a colony of the yeast that contained each different interactor. By this means, each interactor was classified as a strong, medium, or weak binder, which was normalized to the amount of blue color caused by the various naturally-occurring partner proteins of Cdk2 in side by side mating interaction assays. An example of the peptide sequence of one representative of each class is given here:

Detailed Description Text (51):

In related experiments, 6 additional aptamers (i.e., pep6 (SEQ ID NO: 21), pep7 (SEQ ID NO: 22), pep9 (SEQ ID NO: 23), pep12 (SEQ ID NO: 24), pep13 (SEQ ID NO: 25), and pep14 (SEQ ID NO: 26) were shown to interact with the LexA-Cdk2 bait but not with unrelated proteins such as Max or Rb, or with certain Cdk family members such as Cdk4, which shares 47% sequence identity with Cdk2 (FIG. 4A). However, some aptamers interacted with other Cdk family members. The fact that different peptide aptamers showed distinct patterns of cross-reactivity with different Cdks indicated that these aptamers recognized different epitopes conserved among various Cdks. The sequence of the peptide loops is shown in FIG. 4B. Non-unit-length peptides occurred at the same frequency among the Cdk2 interacting aptamers as in the library as a whole. No aptamer showed significant sequence similarity to known proteins, as expected if the 20-mer peptides indeed formed novel recognition structures. All of the peptides were charged, suggesting that some of their interactions with the Cdk2 target could be ionic.

Detailed Description Text (56):

To determine the binding affinities of these aptamers for Cdk2, the following experiments were carried out. Based on interpolation from interaction trap calibration experiments (Estojak et al., Mol. Cell. Biol. 15:5820-5829 (1995)), the robust transcription that some of the aptamers of FIGS. 4A and 4B directed from the pSH18-34 reporter suggested that the equilibrium dissociation constants (Kds) of the interactions was  $<10^{-6}$  M. In order to precisely measure the binding affinity of the aptamers to Cdk2, we used an evanescent wave instrument (BIAcore, Pharmacia, Piscataway, N.J.). Purified His6-Cdk2 was coupled to CM-dextran chips, and peptide aptamers flowed in running buffer over the chips. Following binding, the chips were rinsed with running buffer lacking aptamer.

Detailed Description Text (59):

The ability to select TrxA-peptides that interact specifically with designated intracellular baits allows for the creation of other classes of intracellular reagents. For example, appropriately derivitized TrxA-peptide fusions may allow the creation of antagonists or agonists (as described above). Alternatively, peptide fusions allow for the creation of homodimeric or heterodimeric "matchmakers," which force the interaction of particular protein pairs. In one particular example, two proteins are forced together by utilizing a leucine zipper sequence attached to a conformation-constraining protein containing a candidate interaction peptide. This protein can bind to both members of a protein pair of interest and direct their interaction. Alternatively, the "matchmaker" may include two different sequences, one having affinity for a first polypeptide and the second having affinity for the second polypeptide; again, the result is directed interaction between the first and second polypeptides. Another practical application for the peptide fusions described herein is the creation of "destroyers," which target a bound protein for destruction by host proteases. In an example of the destroyer application, a protease is fused to one component of an interacting pair and that component is allowed to interact with the target to be destroyed (e.g., a protease substrate). By this method, the protease is delivered to its desired site of action and its proteolytic potential effectively enhanced. Yet another application of the fusion proteins described herein are as "conformational stabilizers," which induce target proteins to favor a particular conformation or stabilize that conformation. In one particular example, the ras protein has one conformation that signals a cell to divide and another conformation that signals a cell not to divide. By selecting a peptide or protein that stabilizes the desired conformation, one can influence



whether a cell will divide. Other proteins that undergo conformational changes which increase or decrease activity can also be bound to an appropriate "conformational stabilizer" to influence the property of the desired protein.

Detailed Description Text (63):

Peptide 13 does not affect the growth of a cdc28-1Nts strain at high temperature when the defect is complemented by a plasmid expressing wild-type Cdc28 product, and has no effect on yeast at the permissive temperature. While we do not intend to be bound by any particular theory, it appears that this peptide blocks yeast cell cycle progression by binding to some face of the Cdk2 molecule and inhibiting its function and thereby interfering with its ability to interact with cyclins, other partners, or with substrates.

Detailed Description Text (64):

In later experiments with the aptamers of FIG. 4B, inhibition of Cdk2 activity by these peptides (for example, by binding to a face of the molecule and by blocking its interaction with one of its partner proteins or substrates) was examined. In particular, the ability of the aptamers to inhibit phosphorylation of Histone H1 by Cdk2/Cyclin E kinase was tested. To carry out these experiments, 2.times.10.sup.7 Sf9 cells were co-infected with recombinant baculoviruses expressing hemagglutinin-tagged Cdk2 and His6-Cyclin E as described (Kato et al., Genes & Dev. 7:331-342 (1993); Desai et al., Mol. Biol. Cell 3:571-582 (1992)). Cells were lysed 40 hours after infection in 500 .mu.l of 1.times.Kinase Buffer (Kato et al., supra), and 5 .mu.l of a 100-fold diluted extract was used in 30 .mu.l reactions. Reactions were carried out for 20 minutes at 25.degree. C. by adding 2.5 .mu.Ci of [.gamma..sup.32 P] ATP (3000 Ci/mmol), 25 .mu.M ATP, 100 ng of Histone H1 (Sigma, St. Louis, Mo.), and varying amounts of His6-TrxA or His6-aptamers. Samples were run on 15% SDS-PAGE gels and exposed by autoradiography.

Detailed Description Text (65):

The results of these experiments are shown in FIG. 8. All tested aptamers were able to inhibit phosphorylation of Histone H1 by Cdk2/Cyclin E kinase. Under standard conditions (pH 7.5, 0 mM NaCl) (Kato et al., supra), apparent half-inhibitory concentrations ranged from 1.5 to 100 nM. To rule out the possibility that a trace bacterial contaminant was responsible for the inhibition, we removed the His6-peptide aptamer from the Pep2 preparation with a rabbit polyclonal anti-thioredoxin antiserum; this immunodepleted preparation no longer inhibited Cdk2 kinase activity. Half-inhibitory concentrations of aptamers were lower than the Kds measured from evanescent wave experiments, consistent with the idea that some of the energy of each interaction is ionic and is reduced by the salt in the evanescent wave instrument running buffer.

Detailed Description Text (67):

Previous studies have established that libraries of unconstrained peptides contain sequences capable of recognizing targets in vitro (Devlin et al., Science 249:404-406 (1990); Cwirla et al., Proc. Natl. Acad. Sci. USA 87:6378-6382 (1990); Lam et al., Nature 354:82-84 (1991); Songyang et al., Current Biology 4:973-982 (1994); Scott et al., Current Biology 5:40-48 (1994)) and in yeast (Yang et al., Nucl. Acids. Res. 23:1152-1156 (1995)); such isolated peptide sequences often bear similarity to natural interactors. By contrast, although constrained peptide libraries are less conformationally diverse (McConnell et al., Gene 151:115-118 (1994)), the lack of conformational diversity should lower the entropic cost if binding causes the loop to adopt a single conformation (Spolar et al., Science 263:777-784 (1994)); this reduction in entropic cost may account for the fact that our Cdk2 peptide aptamers recognize their targets with higher affinity than is typically observed for unconstrained peptides (Yang et al., supra; Oldenburg et al., Proc. Natl. Acad. Sci. USA 89:5393-5397 (1992); McLafferty et al., Gene 128:29-36 (1993)). This high affinity suggests that peptide aptamers may inhibit protein function in vivo, in the simplest case by binding to specific faces of the target molecule and disrupting its interaction with specific partners or effectors.

Detailed Description Text (68):

The ability to generate large numbers of aptamers from combinatorial libraries, taken together with the interaction trap, which offers a powerful selection for those that bind specific proteins, facilitates the selection of peptide aptamers against a variety of intracellular targets. Aptamers which inhibit protein contacts can be used to aid the dissection of the networks of protein interactions that govern division of higher eukaryotic cells and can also be used for the genetic analysis of those metazoan organisms for which isolation of specific missense alleles may be impractical. The analogy of the aptamers of the invention with antibodies indicates that peptide aptamers can also be used in other applications in which immunological reagents are now employed, such as ELISAs, immunofluorescence experiments, and sensors. If desired, the affinity of these aptamers may be increased, for example, by increasing their valency and using existing interaction technology to select mutants that bind more tightly. This first generation of peptide aptamers facilitates the production of recognition modules for intracellular nanotechnologies aimed at destroying, modifying, and assembling macromolecules inside cells.

Detailed Description Text (69):3. Thioredoxin Interaction Trap with OncoRas BaitDetailed Description Text (73):

Such mutationally-activated conformational changes in GTP-bound H-ras mutants provide targets for members of a conformationally constrained random peptide library. In the present example, the library is a conformationally constrained thioredoxin peptide library, as described above. Library members, which interact with oncogenic Ras have been identified using a variation of the interaction trap technology provided above. The oncogenic Ras peptide aptamers isolated may be assayed for their ability to disrupt the interaction of oncogenic Ras with known effectors and to inhibit cellular transformation.

Detailed Description Text (79):

Aliquots (50 .mu.l) of the cells were then incubated at 30.degree. C. for 30 min. with 1 .mu.g of thioredoxin peptide library DNA, 70 .mu.g of salmon sperm DNA, and 300 .mu.l of sterile 40% PEG 4000 in LiOAc/TE. The mixtures were heat-shocked at 42.degree. C. for 15 min. Each aliquot was plated onto a 24 cm.times.24 cm plate containing glucose Ura-His-Trp- medium and was incubated at 30.degree. C. for two days. The transforming efficiency typically ranged from 50,000 to 100,000 colony forming units per .mu.g of library DNA.

Detailed Description Text (81):

One .mu.l of each sample was used to transform E. coli KC8 cells by electroporation. Bacterial transformants were selected on minimal agar supplemented with uracil, leucine, histidine, and ampicillin. Each type transformant resulted in final isolation of plasmid which a leucine marker, which carries a DNA fragment encoding thioredoxin-peptide fusion protein.

Detailed Description Text (86):

The protein interaction assays described herein can also be accomplished in a cell-free, in vitro system. Such a system may begin with a DNA construct including a reporter gene operably linked to a DNA-binding-protein recognition site (e.g., a LexA binding site). To this DNA is added a bait protein (e.g., any of the bait proteins described herein bound to a LexA DNA binding domain) and a prey protein (e.g., one of a library of conformationally-constrained candidate interactor prey proteins bound to a gene activation domain). Interaction between the bait and prey protein is assayed by measuring the reporter gene product, either as an RNA product, as an in vitro translated protein product, or by some enzymatic activity of the translated reporter gene product. Alternatively, interactions involving

conformationally constrained proteins may be carried out by direct in vitro techniques, for example, by any standard physical or biochemical technique for identifying protein interactions (such as immobilization of a first protein on a column or other solid support and contact with a conformationally-constrained protein). These direct in vitro approaches are preferably carried out in such a way that the DNA encoding the conformationally-constrained protein may be readily isolated, for example, by using techniques involving phage display or display of the protein on the E. coli flagella.

Detailed Description Text (88):

In one particular embodiment, interacting proteins identified in vitro are tested for their ability to interact in vivo. Such in vivo interacting proteins may be used for any diagnostic or therapeutic purpose. For example, proteins shown to interact in vivo may be used to disrupt, encourage, or stabilize intracellular interactions or may be used as an intracellular antibody-type reagent.

CLAIMS:

2. A method of assaying an interaction between a first protein and a second protein, comprising:

- (a) providing said first protein;
- (b) providing a fusion protein comprising said second protein, said second protein having reduced structural flexibility due to disulfide bonding between cysteine residues at said second protein's amino- and carboxy-termini;
- (c) contacting said first protein with said fusion protein under conditions which allow complex formation;
- (d) detecting said complex; and
- (e) determining whether said first protein interacts with said fusion protein inside a cell by the steps of:
  - (i) providing a host cell which contains (a) a reporter gene operably linked to a DNA-binding-protein recognition site; (b) said first protein covalently bonded to a DNA binding protein which specifically binds to said DNA-binding-protein recognition site; and (c) said second protein covalently bonded to a gene activating moiety; and
  - (ii) measuring expression of said reporter gene as a measure of an interaction between said first protein and said second protein.

4. A method of assaying an interaction between a first protein and a second protein, comprising:

- (a) providing said first protein;
- (b) providing a fusion protein comprising said second protein, said second protein having reduced structural flexibility due to covalent bonding of both the amino and carboxy termini of said second protein to a conformation-constraining protein;
- (c) contacting said first protein with said fusion protein under conditions which allow complex formation;
- (d) detecting said complex; and
- (e) determining whether said first protein interacts with said fusion protein inside a cell by the steps of:

(i) providing a host cell which contains (a) a reporter gene operably linked to a DNA-binding-protein recognition site; (b) said first protein covalently bonded to a DNA binding protein which specifically binds to said DNA-binding-protein recognition site; and (c) said second protein covalently bonded to a gene activating moiety; and

(ii) measuring expression of said reporter gene as a measure of an interaction between said first protein and said second protein.

6. The method of claim 4, wherein said conformation-constraining protein is thioredoxin.

7. The method of claim 4, wherein said conformation-constraining protein is a thioredoxin-like molecule, said thioredoxin-like molecule being characterized by having a three-dimensional structure substantially similar to that of human or E. coli thioredoxin and containing an active site loop.

8. The method of claim 6, wherein said second protein is inserted into the active site loop of said thioredoxin.

9. The method of claim 7, wherein said active site loop has a structure substantially similar to that of human or E. coli thioredoxin or glutaredoxin.

10. A method for detecting a first protein in a sample, comprising

(a) contacting said sample with a second protein having reduced structural flexibility due to covalent bonding of both the amino- and carboxy-termini of said second protein to a thioredoxin-like molecule, said thioredoxin-like molecule having a three-dimensional structure substantially similar to that of human or E. coli thioredoxin and containing an active site loop, said first protein or said second protein being an intracellular protein, said contacting being carried out under conditions which allow said second protein to specifically bind to said first protein and form a complex; and

(b) detecting said complex.

11. The method of claim 10, wherein said thioredoxin-like molecule is thioredoxin.

12. The method of claim 11, wherein said second protein is inserted into the active site loop of said thioredoxin.

13. The method of claim 10, wherein said active site loop has a structure substantially similar to that of human or E. coli thioredoxin or glutaredoxin.

14. The method of claim 10, wherein said second protein is embedded within said thioredoxin-like protein.

17. The method of claim 1, 2, or 4, wherein said first protein or said second protein is an intracellular protein.

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TITLE: Interaction trap systems for detecting protein interactions

DATE-ISSUED: June 4, 2002

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US-CL-CURRENT: 435/6; 435/4, 435/7.1, 435/7.8, 436/501

Full	Title	Citation	Front	Review	Classification	Date	Reference	Examiner's	Supervisor's	Claims	KMIC	Draw De
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US-CL-CURRENT: 435/6; 435/7.1

Full	Title	Citation	Front	Review	Classification	Date	Reference	Substantive	Administrative	Claims	KMIC	Draw De
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Full	Title	Citation	Front	Review	Classification	Date	Reference	Substantive	Administrative	Claims	KMIC	Draw De
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